

⑪ Publication number:

**0 160 571**  
**A2**

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## EUROPEAN PATENT APPLICATION

⑳ Application number: 85303074.0

㉑ Date of filing: 30.04.85

㉒ Int. Cl.<sup>4</sup>: **C 12 N 15/00**  
**C 07 H 21/04, C 07 C 121/20**  
**/(C12N1/20, C12R1:19)**

㉓ Priority: 30.04.84 US 605488

㉔ Date of publication of application:  
06.11.85 Bulletin 85/45

㉕ Designated Contracting States:  
AT BE CH DE FR GB IT LI NL SE

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㉙ Cloning vehicle.

㉚ DNA capable of transforming a cell to allow regulated *in vivo* formation of a protein-producing DNA segment comprising at least two subsegments positioned so that a specified end of one subsegment is adjacent a specified end of the other subsegment; the clonable DNA comprises: a first DNA sequence comprising one subsegment, the specified end of which is adjacent a DNA site for the enzymatic site-specific recombination; a second DNA sequence comprising the other subsegment, the specified end of which is adjacent a second DNA site for enzymatic site-specific recombination, said second enzyme-specific site being remote from said first enzyme-specific site, whereby *in vivo* exposure of a population of the transforming DNA to a selected enzyme system triggers synchronized DNA rearrangement to form the protein-producing DNA segment and

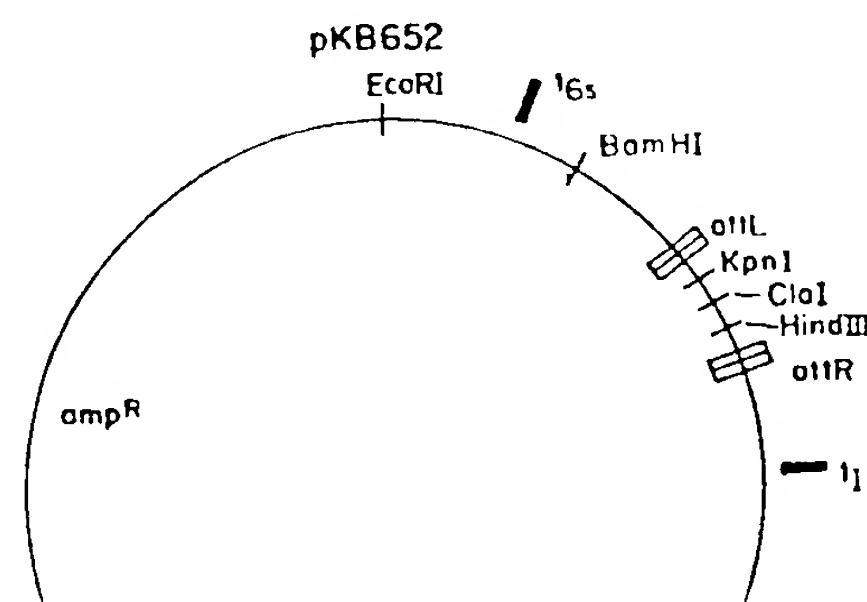


FIG. 1

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CLONING VEHICLE

This invention relates to DNA for transforming cells to enable regulated in vivo protein formation, to the cells that are used in the transformation, to precursors for making such transforming DNA, and to  
5 methods of using the DNA.

It is often useful to engineer an organism with genetic information whose expression is subject to in vivo control. For example, when using engineered organisms to produce a desired compound, it is desirable  
10 to control expression of relevant genes (genes coding for the compound itself, for a compound precursor, or for enzymes in the compound's synthetic pathway) so that compound production is reduced or prevented during the organism's exponential growth phase, because compound  
15 production may be deleterious to the organism or may slow its growth due to energy demands. By controlling compound production in the growth phase, the engineered organism may grow more rapidly and may not be at a competitive disadvantage with respect to nonproducing  
20 organisms (e.g. mutants). Once a satisfactory population of engineered organisms is obtained, they must be made capable of product manufacture. Preferably, such regulatory control is strong; that is, compound production should be dramatically reduced or  
25 eliminated when desired, and compound production should be as copious as possible when desired. Apart from compound biosynthesis it may be desirable to control genetic expression to control other traits of engineered organisms.

Some gene promoters are influenced by regulatory effector molecules which interact with sites at or near the promoter to enable or prevent expression of structural genes under the promoter's control. Thus, expression of a structural gene that has been introduced to an organism may be influenced by engineering the gene to be controlled by such a regulated promoter and then using the regulatory effector molecule to control the gene's expression [Backman et al. (1976) Proc. Nat'l Acad. Sci. USA 71: 4174.].

Other gene promoters are essentially unregulated. These promoters tend to be much stronger (i.e., they induce greater expression of structural genes under their control) than regulated promoters. Until now, however, very strong unregulated promoters have been difficult to use because they cannot be cloned unless followed by an effective signal for termination of transcription and because regulation may be desirable for the reasons discussed above.

Finally, it is known that in certain systems, a DNA segment reversibly inverts, creating a population of organisms that is heterogenous in that in some organisms the DNA segment is in one orientation and in others it is in the opposite orientation. For example, Silverman et al. report an analysis of the genes involved in the DNA inversion responsible for oscillation of flagellar phenotypes of Salmonella, ["Analysis of the Functional Components of the Phase Variation System", Cold Spring Harbor Symposium on Quantitative Biology, 45: 17-26, (Cold Spring Harbor Laboratory, New York, (1981))]. Silverman et al. disclose that the Salmonella DNA

Iino et al. disclose that certain bacteriophage (P1 and Mu) express a DNA inversion factor which is effective to increase the frequency of flagellar phase variation in Salmonella by increasing reversible DNA inversion in that organism, ["Trans-acting Genes of Bacteriophages of P1 and Mu Mediate Inversion of a Specific DNA Segment Involved in Flagellar Phase Variation of Salmonella", Cold Spring Harbor Symposium on Quantitative Biology, 45:11-16, (Cold Spring Laboratory New York, 1981)],

Struhl (1981) J. Mol. Biol. 152:517-533 disclose that the bacteriophage lambda int gene mediates many events including deletion events which randomly generate new gene sequences some small fraction of which may have genetic activity.

#### Summary of the Invention

In one aspect, the invention features, generally, DNA capable of transforming a cell to allow regulated in vivo formation of a protein-producing DNA segment made up of at least two subsegments which are positioned so that a specified end of one subsegment is adjacent a specified end of the other subsegment. In the transforming DNA, the specified end of one subsegment is placed adjacent a first DNA site for enzymatic site-specific recombination, and the specified end of the other subsegment is placed adjacent a second DNA site for enzymatic site-specific recombination, remote from the first enzyme-specific site. At least one subsegment is "exogenous" to (i.e., it does not naturally occur adjacent) its enzyme-specific site. In vivo exposure of a transformed population to a selected enzyme system causes synchronized DNA rearrangement

such formation over enzymatic reversal of the DNA rearrangement which would re-form the subsegments. As used herein, enzymatic site-specific recombination means recombination between DNA sequences at specific DNA sites, which is catalyzed by an enzyme specific for those recombination sites. "Synchronized" rearrangement means rapid rearrangement in a substantial (at least 65%) percentage of the transformed organism population to the desired protein-producing DNA segment, without significant reversal of the rearrangement, that is, re-formation of the subsegments from the desired segment.

In preferred embodiments, the enzyme system is a phage enzyme system for site-specific recombination and the enzyme-specific sites are phage attachment sites. The transforming DNA may be a continuous strand, in which case excision of the DNA between the attachment sites is achieved by using sites having the same absolute orientation; alternatively, inversion of the DNA between the sites is achieved by using sites having opposite absolute orientations. If the transforming DNA is made of two separate strands, then the rearrangement involves integration of the two strands. The complementary enzyme-specific site pair may be attP and attB sites, in which case the enzyme system comprises a phage integration (Int) enzyme; alternatively, the site pair may be attL and attR sites, in which case the enzyme system comprises phage integration (Int) and excision (Xis) enzymes. The two DNA subsegments may be, respectively, a structural gene (including a correctly framed translation initiation sequence) and a promoter for that structural gene in

start signal and the structural gene; these may be extraneous DNA between the promoter and translation start site, for example a recombination site.

Specifically, an unregulated promoter may be used, in  
5 which case the transforming DNA may of necessity include a transcription stop site on the side of the enzyme-specific site opposite from the promoter associated therewith. Alternatively, the DNA subsegments may be portions of the structural gene  
10 coding for a biologically active protein, which portions themselves code for biologically inactive protein segments. Expression of the enzyme system is subject to regulatory control such as the temperature sensitive repressor control of the lambda phage enzyme system.

15 In another aspect, the invention features a method of regulated protein biosynthesis comprising transforming a cell with the above-described transforming DNA, culturing the transformed cell to a desired population of cells which do not produce the  
20 protein, exposing the transforming DNA within the cell population to the enzyme system causing synchronized DNA rearrangement to form the protein-producing DNA segment, and then allowing the cells to synthesize the protein.

In a third aspect the invention features a  
25 precursor to the above-described transforming DNA comprising the two sites for enzymatic site-specific recombination, a restriction endonuclease cleavage site which is unique to the DNA precursor positioned adjacent one of the enzyme-specific sites, and a promoter  
30 sequence positioned adjacent the other enzyme-specific site so that its promoting function is directed toward that site.

The invention enables effective regulation of protein production. One enzyme molecule can rearrange a large number of DNA molecules, so that control exerted over the enzyme is amplified. Moreover, the invention  
5 enables use of promoters which are stronger than those regulated by regulatory effector compounds because the strong promoter can be controlled by a transcription stop sequence until gene expression is desired. The invention improves the efficiency of cell growth and  
10 culturing steps because during cell growth and culturing, the genetic information necessary for the production of the protein is simply not present in a functional form in the cell and therefore cannot be expressed to hinder cell growth. Once protein  
15 production is desired, however, the necessary genetic material is efficiently created.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments and from the claims.

20 Fig. 1 is a diagram of the DNA of plasmid pKB652 showing certain sites thereon;

Fig. 2 is a diagram of the DNA of plasmid pKB686 showing certain sites thereon;

25 Fig. 3 is a diagram of the DNA of plasmid pKB706 showing certain sites thereon;

Fig. 4 is a diagram of the DNA of plasmid pKB730 showing certain sites thereon;

Fig. 5 is a DNA diagram depicting an excision event;

30 Fig. 6 is a DNA diagram depicting an inversion



The two sites shown in the DNA diagrams in Figs. 1-4 which are specific for enzymatic reciprocal recombination are phage attachment sites, labeled respectively attL and attR; that is, they are sites at which a phage enzyme system catalyzes site-specific recombination. Specifically, the pair of sites shown in the figures is functionally equivalent to BOP' (or attL) and POB' (or attR), where O is a region of homology between the chromosome of a bacterial host and an infecting phage, B and B' are bacterial chromosome regions flanking O, and P and P' are phage DNA regions flanking O. Alternatively, the pair BOB' (or att B) and POP' (or att P) could be used. Functional equivalency means the site is recognized by a site-specific recombination enzyme system.

No matter which pair is used, it should be matched to the enzyme system that is to be used to effect the desired rearrangement, so that the system will be driven substantially to complete the rearrangement and to avoid the opposing reverse reaction which forms the DNA fragments from the desired protein-producing DNA sequence. The attL/attR pair is matched to the enzyme system for excision which, in the phage lambda system, comprises both the Int and Xis proteins. The attB/attP pair is matched to the enzyme for integration which, in the phage lambda system, is the Int protein without the Xis protein. Finally, functioning of both the Int and the Int/Xis enzyme systems requires an integration host factor (IHF), a protein produced by the host bacterium.



the transforming DNA and the relative orientation of the enzyme specific sites. As shown in Figs. 5 and 6 excision will be strongly favored if the sites are oriented in the same absolute direction and inversion will be strongly favored if the sites are oriented in the opposite absolute orientation. For example BOP' has the same absolute orientation as POB', and BOP' has the opposite orientation as B'OP.

Integration is the reverse of the process depicted in Fig. 5.

In addition to the enzyme specific sites described above, the transforming DNA includes two DNA subsegments which are to be brought into juxtaposition by the DNA rearrangement. Fig. 4 shows a strong, unregulated constitutive promoter segment as one of the subsegments and a structural gene sequence as the other subsegment. Adjacent the structural gene segment is a translation initiation site, positioned in reading frame with, and on the same side of the recombination site as, the structural gene, so that after rearrangement, the rearrangement site which separates the promoter from the structural gene is not translated into an extraneous amino acid sequence. Thus, after rearrangement, the two subsegments do not necessarily abut one another. In Fig. 4, the promoter is labeled PII to designate the gene II promoter from phage ml3. The structural gene in the transforming DNA codes for T4 DNA ligase from phage T4.

The PII promoter is oriented to promote transcription toward the adjacent attR site, and the T4 ligase gene is oriented so the end to be transcribed

gene.  $T_I$  is a transcription termination site adjacent the attR site on the side of it opposite to the PII promoter.  $T_I$  serves to stop transcription from the unregulated PII promoter, thus preventing wasted cell  
5 energy in unproductive transcription before DNA rearrangement is triggered. Transcription starting at PII and terminating at  $t_I$  is indicated by a wavy arrow. A second transcription termination site, labeled  $t_{6S}$ , is positioned near the attL site to prevent  
10 transacription into and expression of the structural gene prior to DNA rearrangement.

Plasmid pKB730 also includes a gene for ampicillin resistance ( $amp^R$ ) which is a marker for detecting successfully transformed cells.

#### 15 Construction

The construction of plasmid pKB730 is depicted in Figs. 1-3. Unless specifically indicated otherwise, recombinant DNA techniques suitable for the steps described are found in Maniatis et al, Molecular  
20 Cloning, (Cold Spring Harbor Laboratory 1982).

A specific rearrangement vector is constructed to have the following properties: multiple unique cloning sites are bracketed by functional equivalents of lambda attL and attR, and this construction is bracketed  
25 by transcription terminators to terminate transcription into and out of the region in one orientation. The construction is described as follows.

DNA fragments corresponding to the region of the lambda attachment site (HindIII at position 27479 to  
30 BamHI at position 27972) and the region of the 6S transcript (HinIII at position 44141 to EcoRI at position 44972) are separately cloned from bacteriophage

positions referred to herein are those indicated in Sanger et al., (1982) J. Molec. biol. 162:729-773] onto pBR322 [a commercially available plasmid], yielding pKB603 and pKB602 respectively.

5           The following pieces of DNA can be prepared from pKB602, pKB603, or pBR322, and assembled in a series of steps whose particulars are not of consequence to the functioning of the completed assembly:

- 10           1. a piece carrying the 6s transcription terminator  $t_{6s}$ ) extending from a HaeII site within the 6s transcript gene to a Sau3AI site just past the 6s transcription terminator (the HaeII site is converted to an EcoRI site by means of a linker);
- 15           2. a piece carrying a functional equivalent of attL extending from a BamHI site to an AluI site immediately adjacent to the core sequence of attP (the AluI site is converted to a KpnI site by means of a linker);
- 20           3. a portion of pBR322 comprising the ClaI and HindIII sites (the ClaI site is joined to a KpnI linker in a manner which regenerates the ClaI site);
- 25           4. a piece carrying a functional equivalent of attR extending from a DdeI site immediately adjacent to the core of attP to a HindIII site (the HindIII site might be converted to a PvuII site by treatment with nuclease S1, but it is preferable to use PvuII linkers).
- 30

fragment 1 is joined to the EcoRI site of pBR322; the Sau3AI site of fragment 1 is joined to the BamHI site of fragment 2, regenerating a BamHI site. The KpnI site of fragment 2 is joined to the KpnI site of fragment 3. 5 the HindIII site of fragment 3 is joined to the DdeI site of fragment 4; since both ends are filled-in prior to joining, a HinIII site is recreated. The PvuII site of fragment 4 is joined to the PvuII site of pBR322. The resulting plasmid is pKB652 (Fig. 1).

10 A strong constitutive promoter is then cloned into the vector pKB652. In outline, the gene II promoter from a derivative of phage ml3 is cloned by standard methods on a piece of DNA which extends from a Sau96I site to an NdeI site. The nucleotide sequence of 15 ml3 is published by Van Wezenbeck et al. (1980) Gene 11:129-148. The Sau96I end is converted to a ClaI site, and the NdeI end is converted to a HindIII site by use of linkers in a series of steps, and the resulting promoter-containing piece of DNA is cloned between the 20 ClaI and HindIII sites of pKB652, yielding pKB686 (Fig. 2). Alternatively, a variant of pKB686 is made in which the ClaI site is converted to an XhoI site by means of a linker, yielding pKB706 (Fig. 3). The use of either pKB686 or pKB706 may be preferred depending on 25 circumstances related to the structural gene to be cloned for expression, such as cleavage sites available when cloning the gene.

A piece of DNA carrying the gene for T4 DNA 30 ligase is cloned from phage lambda NM989. [Wilson et al (1979) J. Molec. biol. 132:471-491]. In a series of steps, an AluI site separating the gene from its natural promoter is converted to a KpnI site, and the HindIII

The gene is then cloned into pKB706 between the KpnI and XhoI sites. This yields pKB730 (Fig. 4).

Plasmid pKB686 is deposited with the American Type Culture Collection with accession number ATCC 39671. Plasmid pKB706 is deposited with the American Type Culture Collection with accession number ATCC 39672.

The pKB730 cloning vehicle is used to transform E. coli strain KB204, a spontaneous galactose utilizing revertant of derivative of strain M5160] [Greer (1975) Virology 132:471-491 (1979)], which contains on its chromosome a defective lambda prophage with a temperature sensitive repressor controlling production of the int and xis genes. Strain KB204 is deposited with the ATCC with ATCC accession number 39670.

The successfully transformed cells are cultured at temperatures too low to inactivate the repressor (e.g. about 30°C). In that state, no rearrangement occurs, and PII-promoted transcription is blocked by termination site  $t_I$ .

When a desired culture size is achieved, the temperature is increased to inactivate the repressor and thus allow expression of the lambda prophage integration/excision enzymes Int and Xis.

These enzymes rapidly (well within the cell doubling time) trigger the desired DNA rearrangement, in this case excision to form two DNA molecules from one (see Fig. 5). The enzyme system and the specific sites selected decidedly favor excision and do not allow significant reversal of the rearrangement, because the Int/Xis protein combination favors recombination of the

pair. Therefore, the system favors formation of the desired protein-producing DNA segment which includes the PII promoter adjacent the proper end of the T4 ligase structural gene, and such a structure is formed in at least 65% of the transformed cell population. Where the amount of enzyme system is the limiting factor controlling yield of cells with rearranged DNA, yield may be improved by increasing the copy number or promoter strength for the enzyme-system genes in the transformed cells.

In a lysate from a culture in which rearrangement has been induced, after three hours, a large portion ( $>10\%$ ) of the soluble protein is T4 DNA ligase. Upon purification, a small amount of ligase activity is detected in the uninduced lysate. T4 ligase of about 90% purity is obtained from the induced lysate after a single step elution from a phosphocellulose column.

Other embodiments are within the following claims. For example, other site-specific enzyme systems can be used. Other host cells, promoters, and structural genes can be used. Moreover, the subsegments need not be a promoter/structural gene combination but instead can be two segments of a structural gene that codes for a biologically active protein. Neither of the subsegments produces a biologically active protein by itself, but, when combined, the active protein is produced. Thus DNA rearrangement may be used to create the structural gene necessary for activity. The enzyme system can be introduced in other ways, for example by phage based cloning vehicles.

Claims

1. DNA for transforming a cell to enable regulated in vivo formation of a protein-producing DNA segment, said segment comprising at least two subsegments positioned in said DNA segment so that a specified end of the first said subsegment is adjacent a specified end of the said second said subsegment, said transforming DNA comprising:

a first DNA sequence comprising the first said subsegment, said specified end of which is adjacent a first-site for enzymatic site-specific recombination,

a second DNA sequence comprising a second said subsegment, said specified end of which is adjacent a second site for enzymatic site-specific recombination, said second enzyme-specific site being remote from said first enzyme-specific site in said transforming DNA,

at least one of said DNA subsequences being exogenous to its adjacent recombination site,

whereby in vivo exposure of a population of cells descended from a cell transformed with said transforming DNA to a selected enzyme system triggers synchronized DNA rearrangement to form said protein-producing DNA segment and to favor such formation over enzymatic reversal of said DNA rearrangement to re-form said subsegments.

2. The transforming DNA of claim 1 wherein said

attachment sites.



3. The transforming DNA of claim 1 wherein said first DNA sequence is on the same strand of DNA as said second DNA sequence.

5           4. The transforming DNA of claim 3 wherein said first enzyme-specific site has the same absolute orientation as said second enzyme-specific site, and said rearrangement comprises excission of sequences between said first enzyme-specific site and said second  
10 enzyme-specific site.

          5. The transforming DNA of claim 3 wherein said first enzyme-specific site has an absolute orientation opposite to said second enzyme-specific  
15 site, and said rearrangement comprises inversion of DNA between said first enzyme-specific site and said second enzyme-specific site.

          6. The transforming DNA of claim 1 wherein  
20 said first DNA sequence is on a first strand of DNA and said second DNA sequence is on a second strand of DNA, and said rearrangement comprises integration of said first and said second strands.

25           7. The transforming DNA of claim 2 wherein said enzyme system comprises a phage integration enzyme, said first enzyme-specific site is an attP or a functional equivalent thereof, site and said second enzyme-specific site is an attB site or a functional  
30 equivalent thereof.

          8. The transforming DNA of claim 7 wherein

9. The transforming DNA of claim 2 wherein  
said enzyme system comprises a phage integration enzyme  
and a phage excision enzyme, said first enzyme-specific  
site is an attL site or a functional equivalent thereof,  
5 and said second enzyme-specific site is an attR site or  
a functional equivalent thereof.

10. The transforming DNA of claim 9 wherein  
said enzyme system comprises the enzyme Int and the Xis  
10 enzyme of phage lambda.

11. The transforming DNA of claim 9 wherein  
said functional equivalent of attR site contains sites  
for site-specific recombination between HindIII at  
15 position 27479 and DdeI at position 27735 of  
bacteriophage lambda; and wherein said functional  
equivalent of attL site contains sites for site-specific  
recombination between AluI at position 27724 and BamHI  
at position 27972 of bacteriophage lambda.

20 12. The transforming DNA of claim 1 wherein  
said first DNA subsegment comprises a promoter sequence,  
and said second DNA subsegment comprises a structural  
gene sequence.

25 13. The transforming DNA of claim 12 wherein  
said promoter sequence is unregulated and said first DNA  
sequence comprises a transcription termination sequence,  
said first enzyme-specific site being positioned between  
said end of said first DNA subsegment and said  
30 transcription termination sequence.

14. The transforming DNA of claim 13 wherein said promoter is the PII promoter comprising a sequence between the Sau 96 I site and the Nde I site of phage m13.

5           15. The transforming DNA of claim 12 wherein said structural gene sequence codes for T4 ligase.

10           16. The transforming DNA of claim 1 wherein said protein-producing DNA segment comprises a structural gene coding for a biologically active protein, and said first and said second DNA subsegment each codes respectively for a segment of said protein which lacks said biological activity.

15           17. The transforming DNA of claim 1 wherein said enzyme system is subject to regulatory control.

20           18. The transforming DNA of claim 17 wherein production of said enzyme system is temperature regulated.

25           19. The transforming DNA of claim 18 wherein said enzyme system comprises lambda phage enzymes produced under the control of a temperature sensitive repressor.

30           20. The transforming DNA of claim 12 wherein said second DNA subsequence comprises a translation initiation signal adjacent and in frame with said structural gene.

21. A method of regulated protein biosynthesis comprising

providing the transforming DNA of claim 1,  
transforming a cell with said DNA,  
5 culturing said transformed cell to a desired  
population of cells which do not produce said protein,  
exposing said cell population to said enzyme  
system triggering said synchronized DNA rearrangement  
and thus forming said protein-producing DNA segment, and  
10 allowing said cells to synthesize said protein.

22. The method of claim 21 wherein said cells  
are E. coli cells.

15 23. The method of claim 22 wherein said E. coli cells comprise a DNA segment corresponding to the  
genes coding for a phage lambda enzyme system.

24. The method of claim 21 wherein expression  
20 of said enzyme system is subject to regulatory control,  
and said exposing step comprises activating said enzyme  
expression.

25 25. The method of claim 21 wherein said enzyme  
expression is temperature sensitive and said culturing  
is performed at a first temperature at which enzyme  
expression is repressed, and said exposing comprises  
changing said first temperature to a second temperature  
at which said enzyme is expressed.

30 26. The method of claim 24 wherein said cells  
are E. coli cells of strain m5160 or a derivative

27. The method of claim 26 wherein said cells are from the strain deposited with the ATCC having accession no. 39670.

28. A precursor to the transforming DNA of claim 1, said precursor comprising  
a first site for enzymatic site-specific recombination,

5 a second site for enzymatic site-specific recombination remote from said first site,  
a restriction endonuclease cleavage site, unique within said DNA precursor, positioned adjacent said first enzyme specific site, and  
10 a promoter sequence positioned adjacent said second enzyme specific site oriented so that its promoting function is directed toward said second site.

29. The precursor of claim 28 wherein said promoter is an unregulated promoter and said precursor comprises a transcription terminator sequence positioned on the opposite side of said enzyme specific site  
5 opposite from said promoter.

30. The precursor of claim 28 comprising a plasmid deposited with the ATCC having accession no. 39671 or 39672.

31. A cell capable of regulated expression of an enzyme system for site-specific recombination favoring one recombination event over the reverse of  
said event, said cell comprising the transforming DNA of

32. The cell of claim 31 wherein said cell comprises prophage DNA coding for said enzyme system.

33. The cell of claim 31 wherein said cell is from the strain deposited with the ATCC having accession no. 39670.

34. A transforming vehicle to enable regulated in vivo rearrangement of DNA, said transforming vehicle comprising at least two DNA subsegments, a specified end of a first said DNA subsegment being adjacent a first site for enzymatic site-specific recombination, and a specified end of a second DNA subsegment being adjacent a second site for enzymatic site-specific recombination,

at least one of said DNA subsegments being exogenous to its adjacent recombination site.

said specified end of said first subsegment being remote from said specified end of said second subsegment in said transforming vehicle,

whereby in vivo exposure of a population of cells descended from a cell transformed with said transforming vehicle to a selected enzyme system triggers synchronized DNA rearrangement in a manner such that, after said rearrangement,

rearrangement is favored over enzymatic reversal of said DNA rearrangement to re-form said subsegments as they exist in said transforming vehicle.

35. A method of fermentation of a desired product compound comprising,

providing the transforming DNA of claim 34,

transforming a cell with said DNA,

5 culturing said transformed cell to a desired population of cells,

exposing said cell population to said enzyme system triggering said synchronized DNA rearrangement and thus forming said DNA segment, and

10 producing said desired product.

36. A cell capable of regulated expression of an enzyme system for site-specific recombination favoring one recombination event over the reverse of said event, said cell comprising the transforming DNA of claim 34.



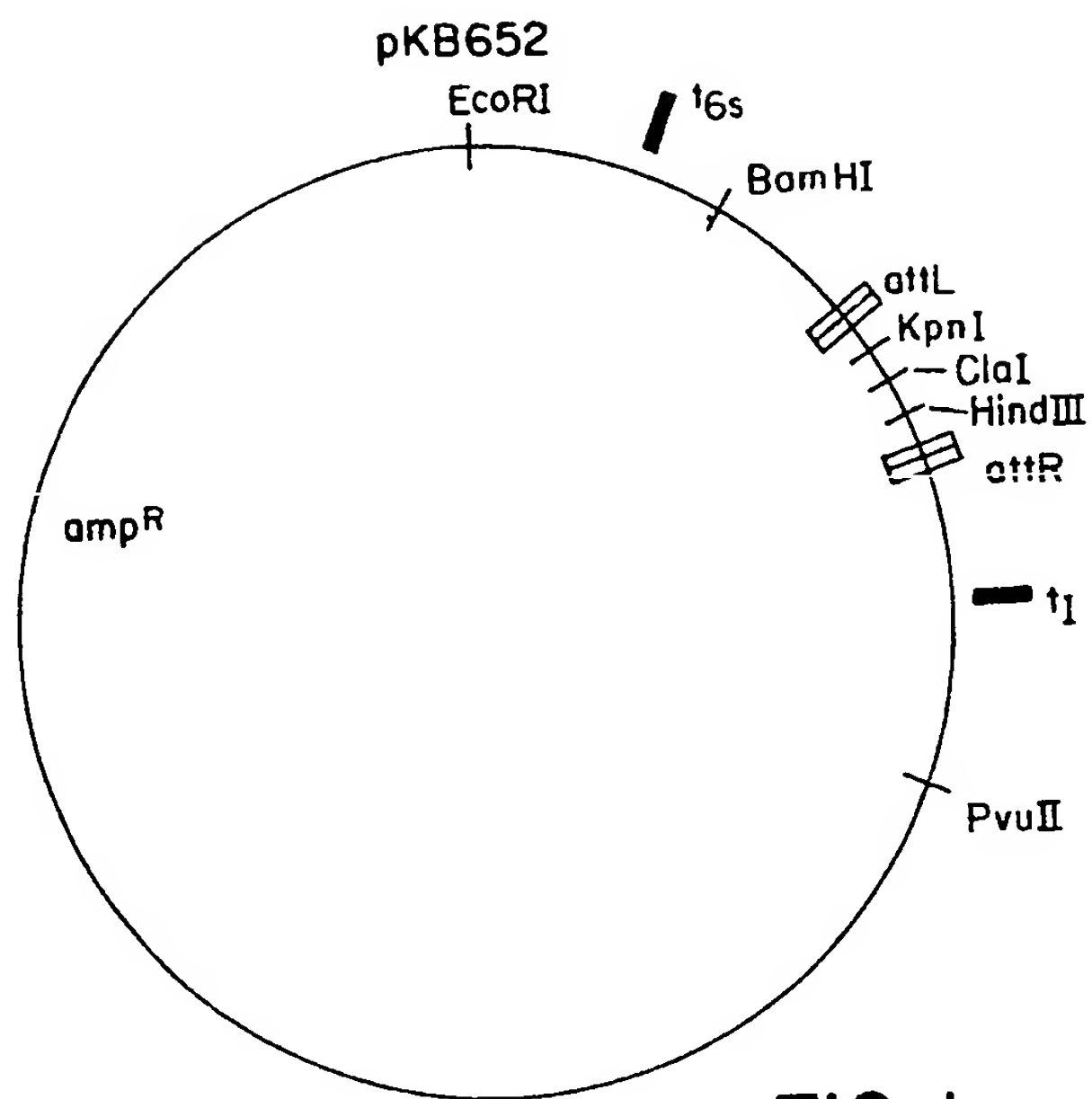


FIG. 1

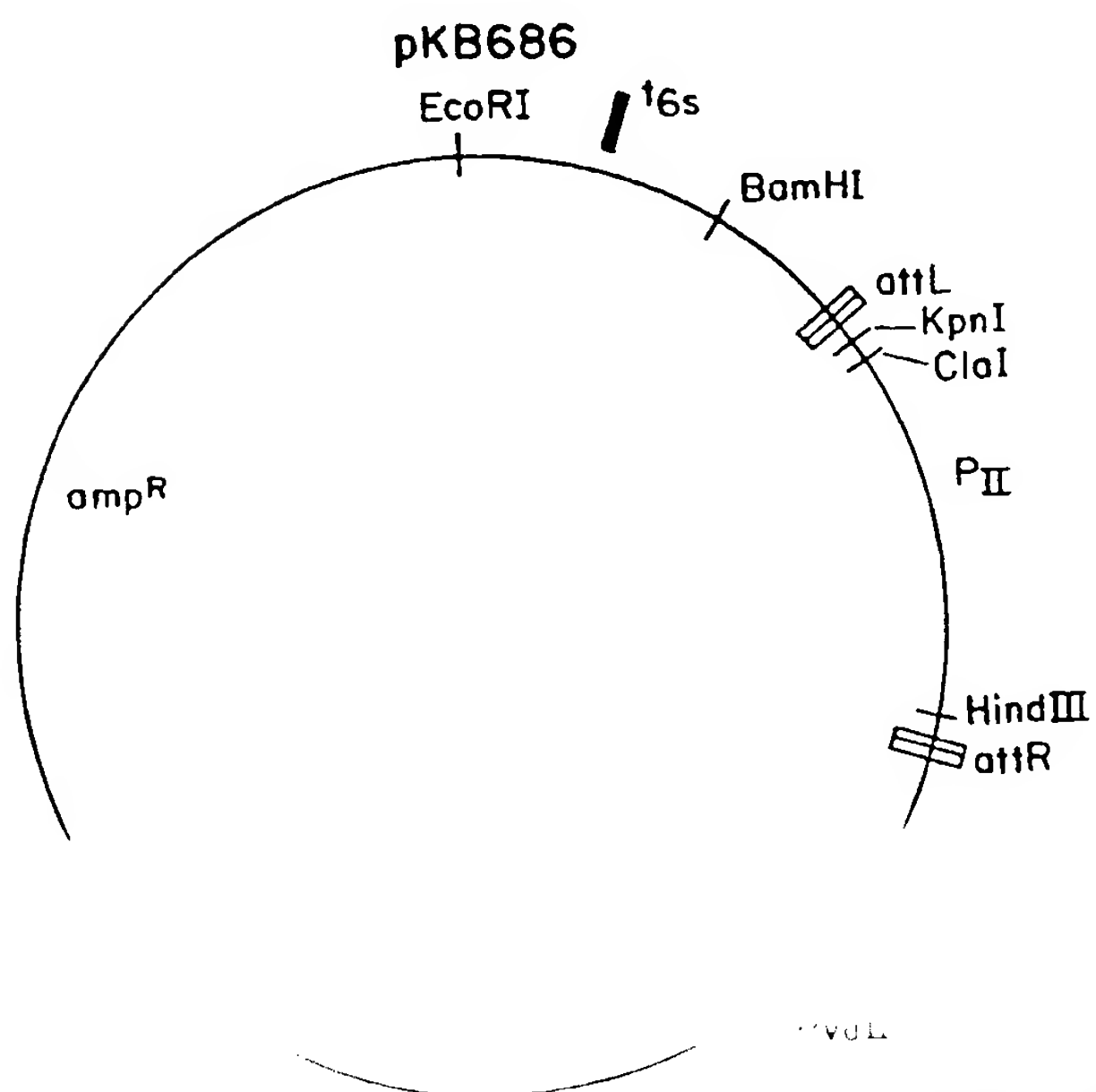


FIG. 2

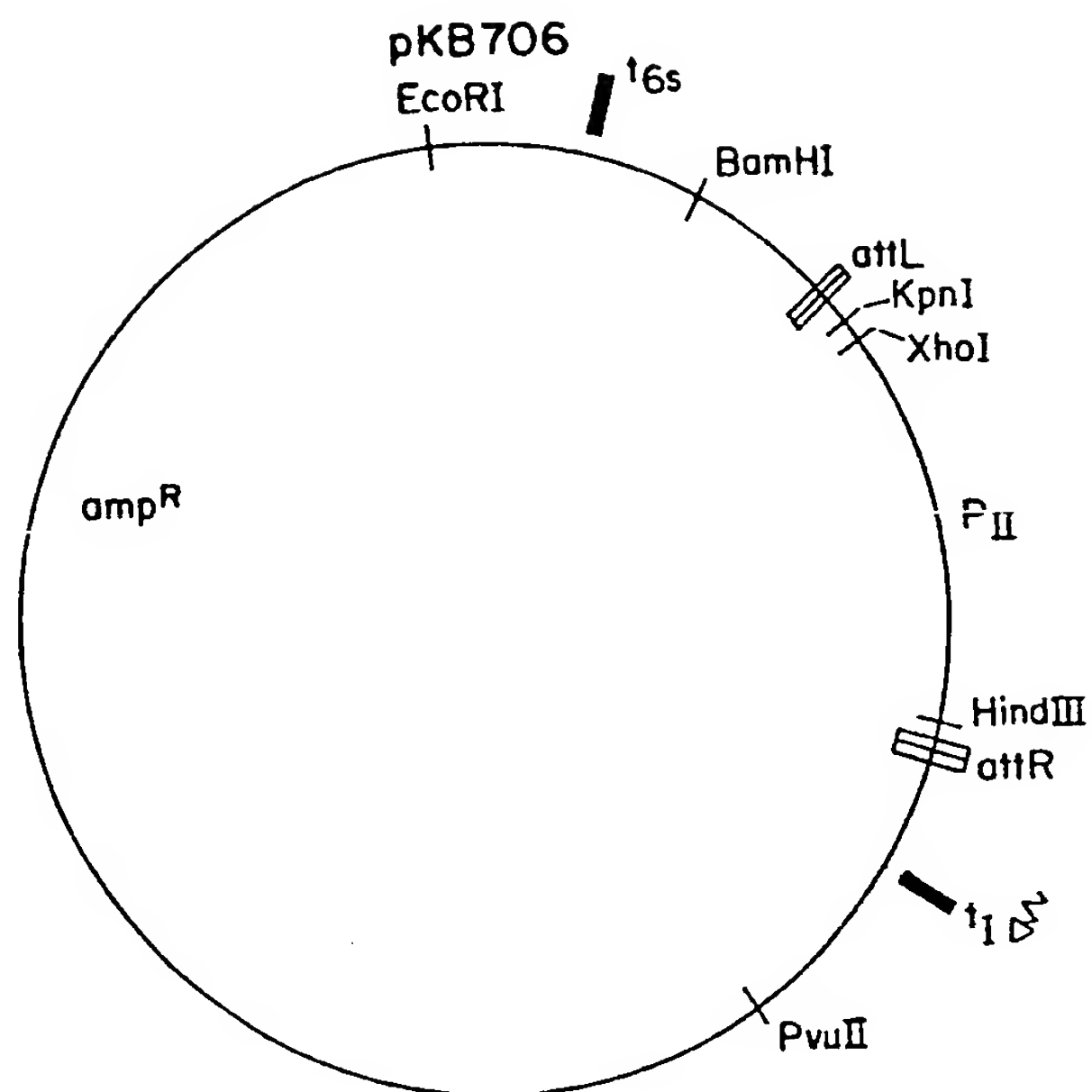


FIG. 3

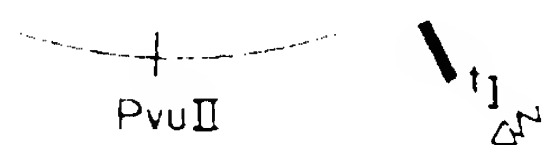
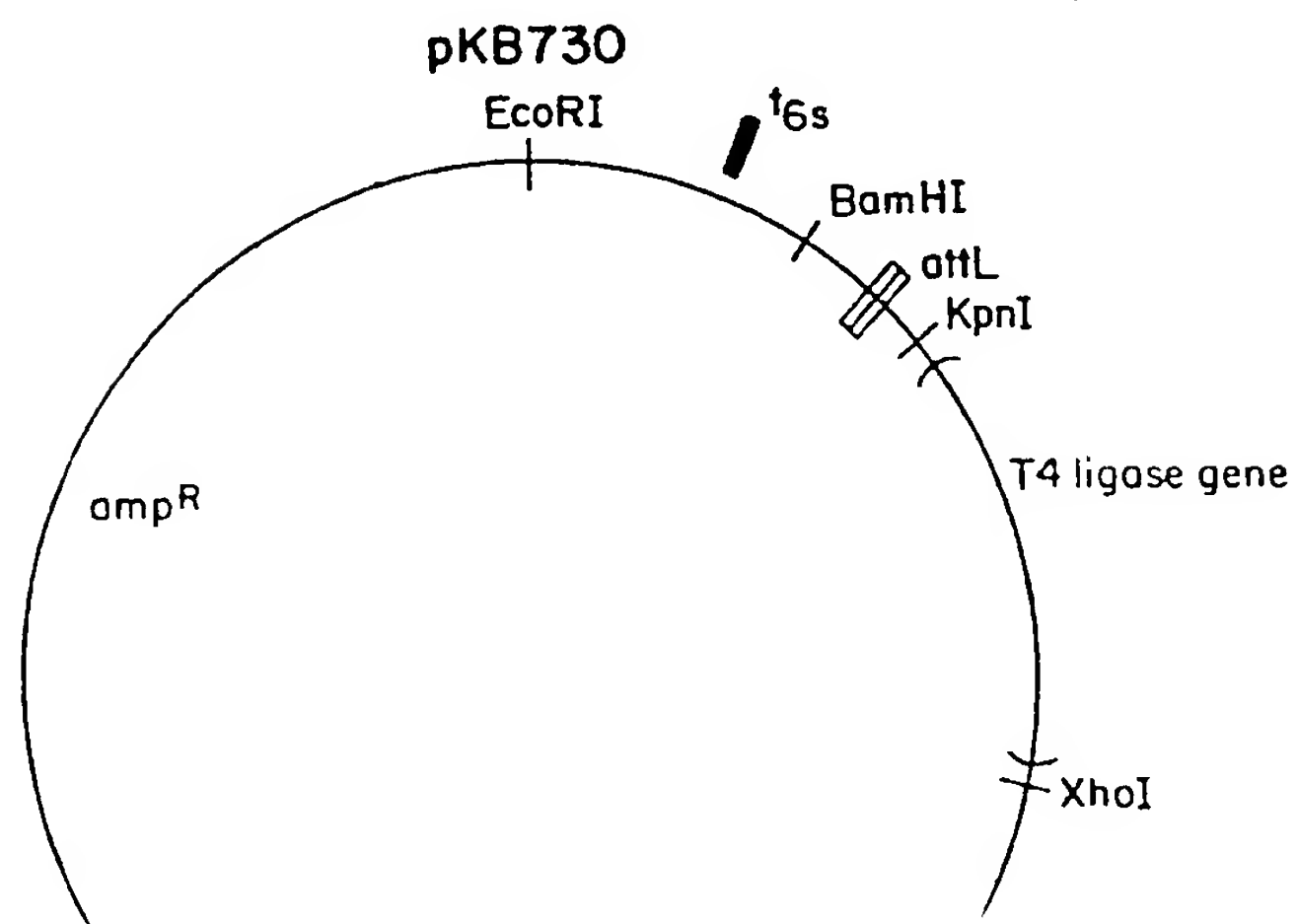


FIG. 4

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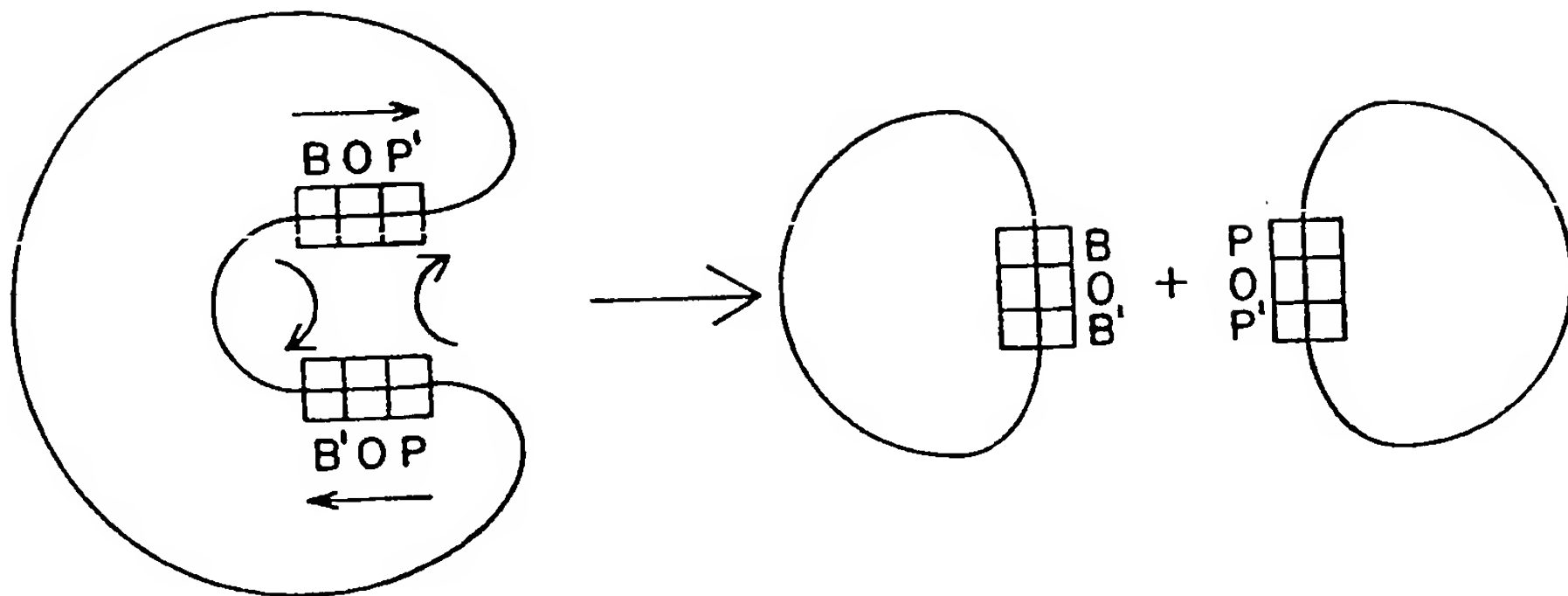


FIG 5

